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Inhibiting protease-activity in peptidomics: strategies during sampling and receptor studies

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INTRODUCTION

Most endogenous peptides are prone to fast metabolization by a broad variety of peptidase and protease enzymes that occur in different tissues and cells. As a result, a main problem in peptidomics analysis is peptide degradation during the sampling step, including sampling storage and sample extraction, aiming at high recoveries for all endogenous peptides present in a sample with no (further) breakdown of the proteins and peptides. Related problems are encountered during peptide functional and receptor binding studies, where protease inhibitors are normally used to improve ligand stability against membrane bound enzymes.

EXPERIMENTAL

A general screening procedure to evaluate peptide metabolic stability and protease inhibitor efficacy is described in Figure 1.

Sample matrices
Typically, plasma, crude tissue/cell homogenates or membrane fractions are used as prepared for peptidomics analyses and *in vitro* experiments such as radioligand binding and permeability studies. The preparation may include a protease denaturation/removal treatment such as microwave heating.

Protease activity testing
Model or target peptides are incubated at pH 7.4 in the sample matrix under examination using a mixing block heater at 37°C. Aliquots are withdrawn at suitable time points, and analyzed (after acidification, heating and centrifugation) using HPLC with PDA and/or fluorescence detection. Results are expressed as half-life times, calculated from the curves obtained.

Protease inhibitor efficacy testing
Peptides are incubated under the same conditions as above, however in absence (*i.e.* reference) and presence of selected protease inhibitors (*e.g.* EDTA, leupeptin). At a pre-determined time point (*e.g.* half-life without protease inhibitors), the reaction is stopped and the mixtures are analyzed as described for protease activity testing. Half-life times are calculated based upon first order kinetics assumption.

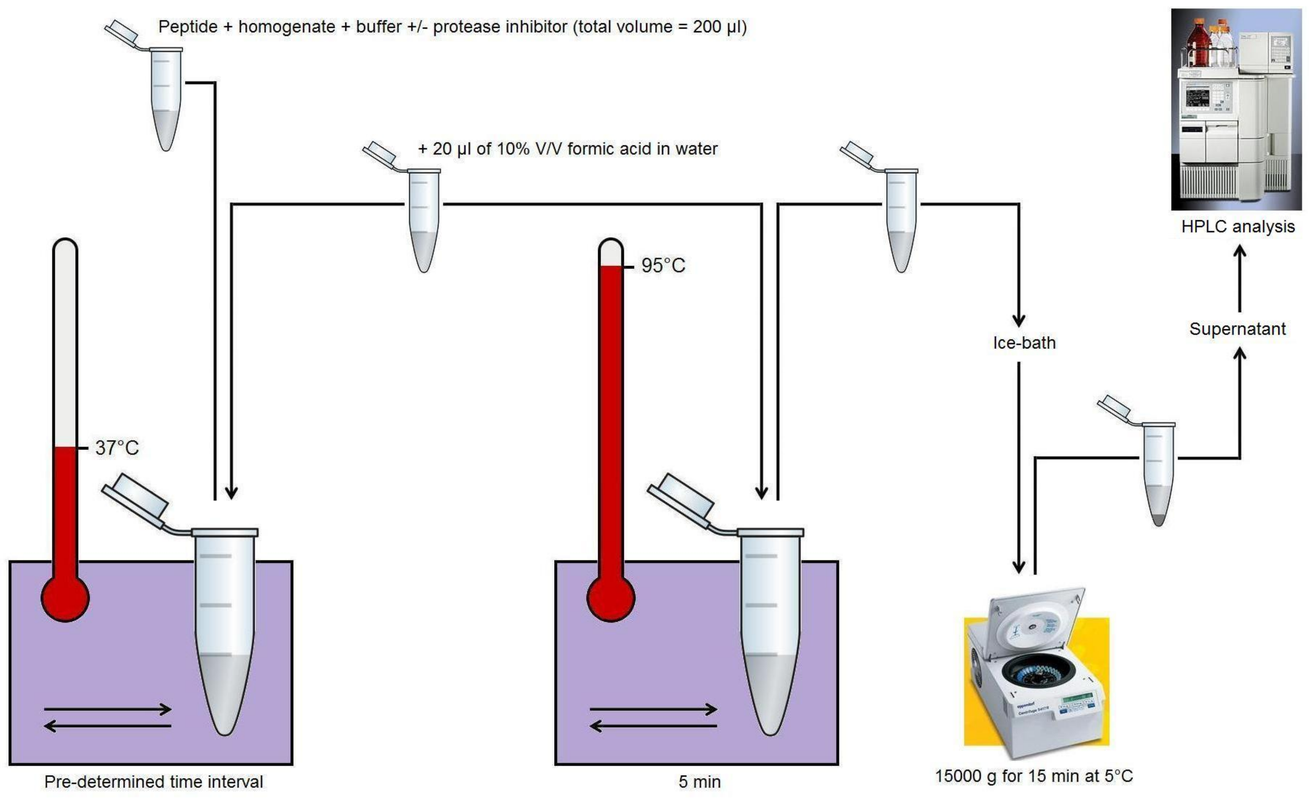

Figure 1. General scheme for peptide stability and protease inhibitor efficacy testing

Typical HPLC conditions: Everest C_{18} 238EV54 (250 mm × 4.6 mm, 300 Å, 5 µm) column (Grace Vydac) at 30°C, with (A) 0.1% formic acid in water, and (B) 0.1% formic acid in acetonitrile. The gradient used for the separation was: 95% A + 5% B from 0 to 5 minutes, followed a linear ramp from 5 to 65 min going to 70% A + 30% B. The flow rate was set at 1.0 ml/min [1].

RESULTS AND DISCUSSION

Typical results are presented here. Other peptides and sample treatments were successfully analyzed using in the same methodology (data not given). Stability of model peptides upon acidification and heat treatment was demonstrated.

The degradation of mouse obestatin in crude mouse brain homogenate (■) and mouse brain membranes homogenate (●), at a peptide/tissue-protein ratio of 1:10 m/m, is described in Figure 2. It was found that this peptide is rapidly degraded when in contact with brain proteases and peptidases. The metabolization half-lives were found to differ by a factor 1.4: 19.3 min and 27.4 min, resp.

Results obtained on protease inhibitors are presented in the Table below. Similarly increased half-lives for both homogenates were observed for EDTA (metal chelator) and a mixture of six protease inhibitors: 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), pepstatin A, E-64, bestatin, leupeptin, and aprotinin.

Protease inhibitor	Half-life (min)			
	Crude brain homogenate		Brain membranes homogenate	
	UV	FL	UV	FL
None (*i.e.* reference)	19	19	27	27
P8340 Protease Inhibitor Cocktail	52	54	74	77
EDTA	38	40	57	67
Soybean Trypsin Inhibitor	20	21	37	40
APMSF	20	21	15	17
1,10-Phenanthrolin	24	24	27	29
Captopril	20	20	27	30
Chymostatin	17	17	28	30

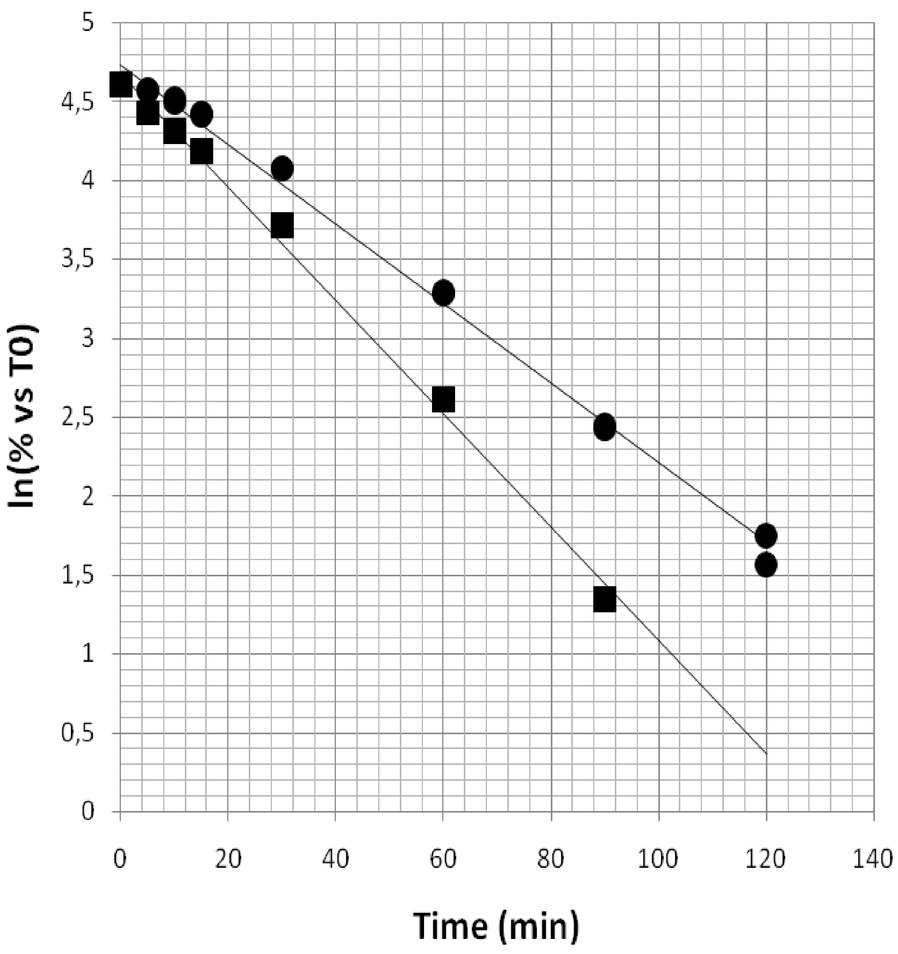

Figure 2. Degradation of mouse obestatin in brain homogenates

CONCLUSIONS

- A simple general method can be applied for (a) testing the susceptibility of peptides to peptidase/protease activity in samples (derived from plasma, tissues and cells), (b) verification of enzyme denaturation in sample preparation protocols, and (c) peptidase/protease activity inhibitor efficacy testing.
- A general protease inhibitor cocktail and EDTA were found improve the metabolic stability of obestatin in brain homogenates.

REFERENCES

[1] V. Vergote, S. Van Dorpe, K. Peremans, C. Burvenich and B. De Spiegeleer. In vitro metabolic stability of obestatin: Kinetics and identification of cleavage products. *Peptides* **29** (2008), pp. 1740-1748.

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